Studying Conformational Dynamics of Amyloidogenic Proteins using Fluorescence Spectroscopy

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Certificate of Examination

This is to certify that dissertation titled “Studying conformational dynamics of amyloidogenic proteins using fluorescence spectroscopy” submitted by Ms. Anubhuti Singh (MS10097) for the partial fulfilment of the BS-MS dual degree programme of the Institute, has been examined by thesis committee duly appointed by institute. Committee finds the work done by candidate satisfactory and recommends that the report be accepted.

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Declaration

The work presented in the dissertation has been carried out by me under the supervision of Dr. Samrat Mukhopadhyay at the Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute.

Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in bibliography.

Date
Anubhuti Singh

In my capacity as the supervisor of the candidate's thesis work, I certified that the above statements by the candidate are true to the best of my knowledge.

Dr. Samrat Mukhopadhyay

(Supervisor)

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STUDYING CONFORMATIONAL DYNAMICS OF AMYLOIDOGENIC PROTEINS USING FLUORESCENCE SPECTROSCOPY

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Abstract:

Protein misfolding leading to amyloid aggregation has attracted considerable interest due to its connection to a range of neurological disorders. It is important to characterize the conformational behaviour of the early oligomeric state of amyloidogenic proteins that serve as precursor to toxic amyloid in order to understand the molecular mechanism of amyloidogenesis. Fluorescence Spectroscopy has been an invaluable tool for the study of biomolecular systems. It is one of the most powerful methods to study protein folding, dynamics, assembly and interactions. In the structural and dynamical studies of proteins, fluorescence spectroscopy is well suited because of its high experimental sensitivity and selectivity to a protein's environment. The present work comprises the study of two amyloidogenic proteins, namely α-synuclein and β_2-microglobulin (β_2m), aggregation of which are involved in Parkinson's disease and dialysis-related amyloidosis, respectively. α–synuclein is an intrinsically disordered protein that is preferentially expressed in presynaptic nerve terminals. It undergoes a large-scale conformational rearrangement upon binding to synaptic vesicle membranes. In order to obtain the structural insights into the membrane-bound α-synuclein in the residue specific manner, we incorporated single cysteine (Cys) at various positions along the sequence. These Cys mutants were labelled with an extrinsic fluorophore, IAEDANS and were used as site-specific fluorescence marker to characterize the dynamical aspects of α-synuclein. On the other hand, β_2m has a classical β-sandwich fold comprising seven antiparallel β-strands and is a component of the major histocompatibility complex class I. Here we have focused on conformational states of β_2m that would be involved in unfolding process as an intermediate state using a host of fluorescence spectroscopic tools. These tools allowed us to monitor the conformational changes of β_2m during its unfolding process.

Chapter 1

Introduction

1. Basic Theory:

Protein misfolding, aggregation, and amyloid fibril formation are associated with a number of human disorders that include Alzheimer's, Parkinson's, Huntington's, and prion diseases as well as type II diabetes and dialysis-related amyloidosis.[1-4] Understanding the molecular mechanism of amyloid formation would represent an important step in elucidating the conformational behaviour of the early oligomeric state of amyloidogenic proteins. Various studies on amyloidogenic proteins have indicated that the presence of intrinsically disordered or partially unfolded structure in the polypeptide chains plays a pivotal role in initiating the process of amyloid assembly.[5-11] There is an emerging consensus that the conformational properties of amyloidogenic disordered polypeptides are in sharp contrast to those of the prototypal denatured state of proteins found in high concentration of chemical denaturant. The mechanism of amyloidogenesis for intrinsically disordered proteins (IDPs) and natively folded proteins are in contrast. In case of IDPs, disorderedness is a necessary condition for aggregation. It has been shown that a very small change in the environment of such proteins often might cause their partial folding and aggregation.[12] Whereas, in case of natively structured proteins, partial unfolding is believed to be a prerequisite for the proteins' assembly into amyloid fibrils.[13] Thus, to understand the molecular mechanism of amyloidosis, it is necessary to find factors that induce partial unfolding in natively structured proteins as well as partial folding in IDPs and subsequent amyloid fibril formation.

Present work involves the study of structural and conformational dynamics of two amyloidogenic proteins, namely human α-synuclein and human β_2-microglobulin (β_2m), aggregation of which are involved in Parkinson's disease and dialysis-related amyloidosis, respectively. Alpha-synuclein (α-synuclein) is a small (14kDa, 140 amino acids), highly acidic, intrinsically unstructured protein that is expressed predominantly in the human brain and concentrated in presynaptic nerve terminals. Structurally, α-synuclein sequence is divided into three distinct regions (Figure 1(a)): N-terminal (1-60 amino acid) which has an affinity to bind to the membranes[14], central region (61-95 amino acid) known as NAC region (non-amyloid component of Alzheimer disease

amyloid) which initiates the aggregation[15]and the third region C-terminal (96-140 amino acid) is highly negatively charged and it facilitates the binding of calcium and other ions.[16] The exact function of α-synuclein protein is poorly understood, though, there are few proposed functions known such as synaptic transmission[17], synaptic vesicle localization[18], and maintenance of neuronal plasticity[19] etc. Furthermore, the N-terminal domain of α-synuclein includes 7 imperfect 11-residue repeats, each containing a variant of the consensus 6-residue sequence KTKEGV, which are similar to repeats found in the exchangeable apo-lipoproteins and are consistent with a class A2 amphipathic α-helices[20] suggesting a lipid-binding activity for N-terminal domain. α-synuclein was shown to associate with synaptic vesicles [21] and to bind to synthetic containing negatively-charged phospholipids.[22] Many biophysical experiments have been performed to understand the interaction between membrane and α-synuclein, the results showed that α-synuclein undergoes a conformational change from random coil to α-helical structure.[23] But the in-depth residue-specific information of the membrane bound α-synuclein is still remains elusive. Therefore, in order to understand conformational transitions of α-synuclein in residue specific manner, we have employed time-resolved fluorescence anisotropy of an environment-sensitive fluorophore, 5-((((2-iodoacetyl) amino) ethyl) amino) naphthalene-1-sulfonic acid (IAEDANS), attached to specific locations in the protein, as a readout. Single cysteine mutant of α-synuclein (A90C) was used for studies. The protein was labelled with IAEDANS, a thiol-labelling fluorescence probe. The site-specific fluorescence anisotropy decay of labelled protein was monitored to probe the conformational dynamics of α-synuclein upon binding to membranes. The results show that the NAC region (Cys 90) is highly structured as indicated by high anisotropy and also showed different degree of structural organization.

β_2-microglobulin (β_2m) is a light chain of the class I major histocompatibility complex (MHC), which is non-covalently attached to the α-chain of the MHC I molecule. Amyloid fibril formation of β_2m is implicated in dialysis-related amyloidosis, a disease reported among those patients who receive prolonged hemodialysis.[24] It is a 99 amino acid residue protein, comprising seven β-strands with a disulfide bond between Cys25 and Cys80, and contains two tryptophan residues at positions 60 and 95 of the polypeptide chain (Figure 4).[25, 26] The tryptophan residue at position 60 is largely exposed, whereas that at position 95 is partially buried.[26] Previous studies have shown

that the transition of β_2m from soluble state to insoluble aggregates can be triggered at low pH with accumulation of distinct intermediate states.[27, 28] A population of a partially unfolded intermediate, possessing the characteristics of a molten globule-like state, builds up at pH 3.6, as also supported by nuclear magnetic resonance (NMR) studies.[27] In the present investigation, we directed our efforts to understand the mechanism of GdmCl-induced unfolding of β_2m using a diverse array of fluorescence spectroscopic tools that allow us to monitor conformational dynamics and chain dimension in the low protein concentration regime. Our fluorescence studies provide an important dynamic signature of an intermediate state of β_2m during the unfolding process that is different from that of the native state and canonical unfolded states of proteins.

2. Experimental Section:

2.1 Materials:

LB (Luria Broth mixture powder), agar powder, Tris, Glycine, SDS (sodium dodecyl sulphate), NaCl (sodium chloride) were purchased from Hi-Media. EDTA (ethylenediaminetetraacetic acid), protease inhibitor cocktail, HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid), DTT (dithiothreitol), Dimethylsulfoxide (DMSO) were purchased from Sigma and used as received. Ampicillin, chloramphenicol and IPTG (isopropyl beta-D-1-thiogalactopyranoside) were purchased from Goldbio.Com. HCL (hydrochloric acid), $CaCl_2$ (calcium chloride), ethanol, glacial acetic acid and ammonium sulphate were purchased from Merck. Q (quaternary ammonium) sepharose fast flow resin was purchased from GE Healthcare and streptomycin sulphate was purchased from CDH and used as received. Chloroform solutions of POPG (1-palmitoyl-2-oleoyl-snglycero-3-phospho (1'-rac-glycerol)) were purchased from Avanti Polar Lipids. Guanidium hydrochloride (GdmCl), 5-((((2-(iodoacetyl) amino) ethyl) amino) naphthalene-1-sulfonicacid) (IAEDANS) and dithionitrobenzonate (DTNB) were purchased from Sigma.

2.2 Protein Expression and Purification

2.2.1 α-synuclein: Cysteine mutants of α-synuclein were purified using a reported protocol. The pT7-7 plasmid with α- synuclein gene was kindly provided by Prof. Vinod

Subramaniam from the University of Twente, The Netherlands. It was transformed in BL21 (DE3) strain of Escherichia coli. Briefly, 1% of the overnight grown culture (containing 100 µg/ml) ampicillin and (35 µg/ml) chloramphenicol of BL21 (DE3) was transformed into fresh media (containing 100 µg/ml ampicillin) and when O.D at 600nm reached to 0.6 – 0.8, the cells were induced with 800 µM IPTG for 4 hours. To obtain the cell pellet, culture was centrifuged at 4,000rpm for 30 min at 4 °C. Pellet was resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 8.0 containing 50 µL protease inhibitor cocktail) and stored at -80 °C till further use. The lysed cells were boiled at 95 °C for 30 min followed by centrifugation at 12,000 rotation per minute (rpm) for 30 min at 4 °C. The supernatant was collected and thoroughly mixed with 136 µL/mL of 10% streptomycin sulphate and 228 µL/mL of glacial acetic acid followed by centrifugation at 12,000 rpm for 30 min at 4 °C. To the clear supernatant, equal volume of saturated ammonium sulphate was added and kept at 4 °C with an intermittent mixing for an hour. The precipitated protein, separated by centrifugation at 12,000 rpm for 30 min at 4 °C was suspended in equal volume of 100 mM ammonium acetate and ethanol followed by centrifugation at 4,000 rpm for 10 min at 4 °C. Finally, the pellet was washed with absolute ethanol and dried at room temperature, until ethanol evaporated. The pellet was suspended in equilibrating buffer (10 mM Tris, pH 7.4) and further purified by FPLC (fast performance liquid chromatography) on a Q Sepharose and the protein was eluted at ~ 300 mM NaCl. The purity of the collected fractions was assessed by SDS- PAGE (SDS- polyacrylamide gel electrophoresis) (Figure 1(b)). The pure fractions were dialyzed in a dialysis duffer (10 mM HEPES, 50 mM NaCl, pH 7.4) and stored at -80 °C.

2.2.2 Human β_2-microglobulin (β_2m):

pET-23a plasmid harbouring mutant of human β_2m gene (W60 & W60 N terminal Cysteine) was expressed in Escherichia coli BL21 DE3-lysogen cells. Briefly, 1% of the overnight grown culture (containing 100 µg/ml ampicillin and 35 µg/ml chloramphenicol) of BL21 (DE3) was transformed into fresh media (containing100 µg/ml ampicillin) and when O.D at 600nm reached to 0.6 – 0.8, the cells were induced with 1 mM IPTG for 8-10 hours. To obtain the cell pellet, culture was centrifuged at 4,000 rpm for 30 min at 4 °C. Pellet was resuspended in lysis buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The lysed cells were sonicated (Amplitude= 20, pulse on time= 10sec for 30 min) followed by

centrifugation at 12,000 rpm for 30 min at 4 °C and inclusion bodies were stored at -80 °C for further use. The inclusion bodies were resuspended in 8 M urea and kept in 4 °C overnight. In soluble material was removed by centrifugation and the solubilized β_2m was then refolded by dialysis into dialysis buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at room temperature. Dialysed protein was purified on an anion exchanger (Q-Sepharose from GE) column, by performing fast flow protein liquid chromatography (FPLC). Fractions showing single peak were separately pooled and further purified using a Superdex-75 column (GE). Stock of solution of purified recombinant protein was stored at 4 °C.

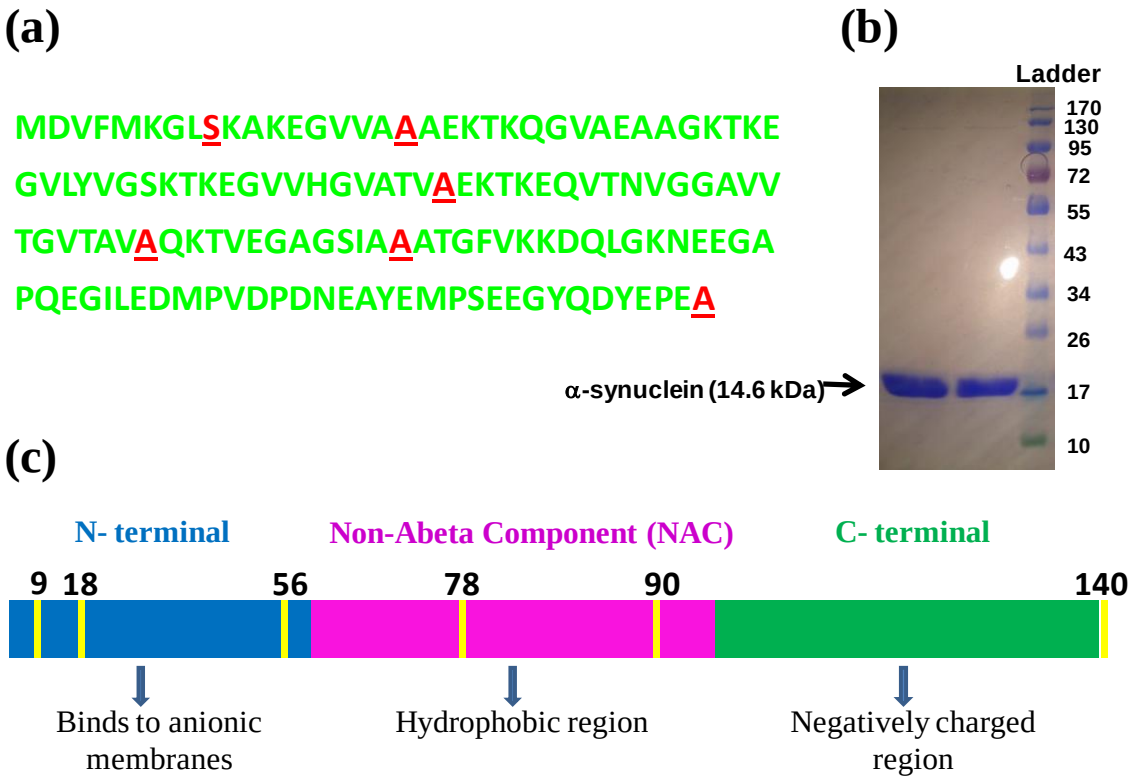

Figure1. (a) Sequence of α-synuclein showing distribution of charged amino acids. Residues underlined were replaced by cysteine (b) SDS-PAGE showing fractions of pure α-synuclein (c) Different regions of α-synuclein with mutant positions highlighted in yellow.

2.3 Preparation of protein sample:

2.3.1 α-synuclein:

Prior to every experiment the single cysteine mutants of α- synuclein were passed through 50 kDa molecular weight cut off (MWCO) Amicon filter (purchased from Milipore) and concentrated using 3 kDa MWCO Amicon. The concentration of protein

was estimated by measuring absorbance using UV-Vis spectrophotometer (Chirascan, Applied photophysics). The concentration of all the mutants was determined using E_{275} = 10,810 M^{-1} cm^{-1}. The measurements were carried out using 1 mm path length cuvette with a scan range of 260-300nm and a scan rate of 1 nm/s. The final spectra averaged over 2 scans and buffer subtracted. The purified proteins were stored at -80 °C.

2.3. β_2-microglobulin:

Prior to every experiment the single tryptophan and N-terminal cysteine mutant of β_2m were concentrated using 3 kDa molecular weight cut off (MWCO) Amicon filter (purchased from Milipore). The concentrations of proteins were estimated by measuring tryptophan absorbance using UV-Vis spectrophotometer (Chirascan, Applied photophysics). The concentration of all the mutants was determined using E_{280} = 14,565 M^{-1} cm^{-1}. The measurements were carried out using 1mm path length cuvette with a scan range of 240-350 nm and a scan rate of 1 nm/s. The final spectra averaged over 3 scans and buffer subtracted. The purified proteins were stored at -80 °C.

For unfolding experiments, protein was incubated in different concentrations of GdmCl ranging from zero to 4 M for 12 hour, and the equilibrium fluorescence signals were measured.

2.4 Fluorescence labelling of proteins:

2.4.1 α-synuclein with IAEDANS:

The labelling of the free thiol group in denatured α-synuclein was carried out in 10 mM HEPES buffer and 50mM NaCl, pH 7.4. Initially, 50 µM of α-synuclein was prepared in 6 M GdmCl kept on spinning rotor for 2 hour at 10 rpm at 25 °C. Approximately 30 equivalents of IAEDANS, dissolved in dry DMSO, were added into the denatured and reduced α-synuclein and the reaction mixture was gain kept on rotor spin for 3 hour at room temperature. After the labelling reaction was complete, the labelled protein was dialyzed in a dialysis buffer (10mM HEPES, 50mM NaCl, pH 7.4) overnight and then concentrated using 3 kDa MWCO Amicon, whereby the free, unreacted dye was removed. Protein concentration was checked by measuring the absorbance at both 280 and 337 nm. The concentration of the labelled protein was determined by the subtracting

the absorption contribution of AEDANS at 280 nm. The molar extinction coefficients of IAEDANS at 280 nm and 337 nm are 12800 M^{-1} cm^{-1} and 6100 M^{-1} cm^{-1}, respectively.

2.4.2 β_2m (W60-N Terminal cysteine) with DTNB:

The labelling of the free thiol group in denatured β_2m was carried out in 6M GdmCl and 20 mM phosphate buffer, pH 7. Initially, 500 µM of β_2m was prepared in 6 M GdmCl to with an aqueous solution of 2 mM DTT was added. The resulting solution was kept on spinning rotor for 2 hour at 10 rpm at 37 °C and then in 4 degree for 3 hour. PD-10 de-salting column was used to refold the protein and to remove DTT. Just after removal of DTT, protein was again refolded using PD-10 in 6M GdmCl and labelled with approximately 100 equivalents of DTNB, dissolved in 6 M GdmCl. The reaction mixture was again kept on rotor spin for 3 hour at room temperature. After the labelling reaction was complete, the labelled protein was dialyzed into a dialysis buffer (10 mM Tris, 1 mM EDTA, pH 8.0) overnight and then concentrated using 3 kDa MWCO Amicon. PD-10 column was used to remove the free dye. Protein concentration was checked by measuring the absorbance at both 280 and 325 nm. The concentration of the labelled protein was determined by the subtracting the absorption contribution of TNB at 280 nm.

2.5 Lipid vesicle preparation:

Liposome's were made from anionic POPG using a reported protocol. Briefly, appropriate amount of the respective chloroform solution was taken in around bottom flask and purged with a gentle stream of nitrogen for 1 hr followed by vacuum desiccation for 2 hours to ensure complete removal of the residual organic solvent. The dried lipid film was hydrated in DPBS (Dulbecco's phosphate buffer saline: 2.67 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl and 8.06 mM Na_2HPO_4, pH 7.4) buffer with intermittent vortexing for an hour to a final lipid concentration of 10 mM. This resulted in a turbid solution having MLVs (multilamellar vesicles). The MLVs were subjected to freeze-thaw cycles, alternating between liquid nitrogen and water bath (preset at 42 °C) for one minute.

2.5.1 Preparation of Small Unilamellar vesicles (SUVs):

The above MLVs were then sonicated for an hour at 40 °C using 37 Hz pulse rate to obtain SUVs. The size of the SUVs (30 nm ± 10 nm) was confirmed by DAWN 8 Helios MALS system (Wyatt Technology).

2.5.2 Preparation of Large Unilamellar vesicles (LUVs):

The above MLVs were subjected to extrusion method in order to prepare LUVs (Mini Extruder, Avanti Polar Lipids). The size of the LUVs (100 nm ± 10 nm) was confirmed by DAWN 8 Helios MALS system (Wyatt Technology).

2.6 Circular Dichroism (CD) experiments:

The CD spectra were collected on Chirascan CD spectrometer (Applied Photophysics, UK) using a 1 mm path length quartz cuvette at room temperature. The concentrations of mutants of α-synuclein and β_2m were fixed as 20 μM. α-synuclein CD spectra in the absence and presence of POPG (1 mM) in DPBS buffer were collected with a scan range of 1 nm/s and averaged over 3 scans. The scan range was fixed from 200 nm to 260 nm. All the spectra were buffer subtracted and smoothened using Chirascan 'ProData viewer' software provided with the instrument.

2.7 Steady-state fluoresecnce measurements:

All steady-state fluorescence measurements were carried out on HORIBA Scientific Fluoromax-4 spectrofluorimeter. The steady-state anisotropy is giving by:

$$r_{ss} = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + 2GI_{\perp}) \quad (1)$$

r_{ss} was obtained from the parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) intensity components with G-factor correction. For α-synuclein, the final concentration of protein and lipids were 50 μM and 2 mM, respectively in all experiments. For AEDANS fluorescence intensity measurements, λ_{ex} and λ_{em} were set to 295 nm (bandpass 1.0 nm) and 350 nm (bandpass 5.0 nm), respectively. The path length of the cuvette was 2 mm. Fluorescence intensity were recorded with integration time of 3 s. AEDANS fluorescence anisotropy was measured by setting λ_{ex} and λ_{em} to 295 nm (bandpass 1.0 nm) and 350 nm (bandpass 5.0 nm), respectively. Integration time of 3 s was used to obtain a satisfactory signal-to-noise ratio.

For β_2m, the final concentration of protein was 20 µM in all experiments. For Trp fluorescence intensity, λ_{ex} and λ_{em} were set to 295 nm (bandpass 0.8 nm) and 350 nm (bandpass 3.0 nm), respectively. Integration time of 1 s was used to obtain a satisfactory signal-to-noise ratio.

2.7.1 FRET (Fluorescence resonance energy transfer):

For fluorescence resonance energy transfer (Trp → TNB) measurements, the concentration of β_2m mutant (W60 N-terminal cysteine) was kept constant in increasing concentration of GdmCl, pH 7.0, as 20 µM. A cuvette (from Hellma) of path length 10 mm x 2 mm was used for the measurements. The tryptophan (Trp) decay in the absence and presence of labelled protein were collected and analyzed. The FRET efficiencies (E) were estimated from the fluorescence intensity of Trp in the absence (F_D) and in the presence of acceptor (F_{DA}) using following relationship.

$$E = 1 - (F_{DA} / F_D) \quad (2)$$

2.8 Time–resolved fluorescence measurements:

The time –resolved fluorescence decays of the samples (in the absence and presence of cysteine variants labelled with IAEDANS of lipids) were collected using a time correlated single-photon-counting (TCSPC) setup (Fluorocube, Horiba Jobin Yvon, NJ). 375 nm LD (Laser Diode) was used as excitation source having repition rate of 1 MHz. All the decays were collected at magic angle (54.7) with 12 nm bandpass and a PhotoMultiplier Tube (PMT) (Hamamastu Corp) was used as detector. An aqueous solution of 2 % Ludox was used to collect the instrument response function (~ 200 ps). In order to obtain a good signal-to-noise ratio, 10,000 counts were collected at the peak. All the experiments were carried out at room temperature.

2.8.1 AEDANS fluorescence intensity decay:

For all the life time measurements, the concentration of α-synuclein mutaunts and SUVs of POPG were kept constant in DBPS buffer, as 50 µM and 2 mM respectively. Cuvette (Hellma) of pathlength 10 mm x 2mm was used. Tryptophan decay was collected in the presence and in the absence of α-synuclein variants at 520 nm. The intensity decay were collected and analyzed.

2.9 AEDANS fluorescence anisotropy decay:

Time-resolved anisotropy decay measurements of the samples were made using a time-correlated single-photon-counting (TCSPC) setup (Fluorocube; Horiba Jobin-Yvon, NJ). The samples were excited using a 375 nm laser-diode (LD). The instrument response function (IRF) at 375 nm was collected using a aqueous solution of 2 % Ludox. The width of the IRF was ~ 200 ps. For the anisotropy decay measurements, the emission data were collected at 0 and 90 with respect to excitation polarization. The emission monochromator was fixed at 520 nm with a bandpass of 20 nm. The anisotropy decays were analyzed by globally fitting $I_{\parallel}$ and $I_{\perp}$ as follows:

$$I_{\parallel} = I(t)[1+2r(t)]/ 3 \quad (3)$$

$$I_{\perp} = I(t)[1-r(t)]/ 3 \quad (4)$$

The perpendicular component of the fluorescence decay was corrected for the G-factor of the spectrometer. I(t) is the fluorescence intensity collected at the magic angle (54.7) at time t. the anisotropy decays were analyzed using a bi-exponential decay model describing fast and slow rotational correlation time as follows:

$$r(t)=r_o[\beta_{fast} \exp(-t/ \varphi_{fast}) +\beta_{slow} \exp(-t/ \varphi_{slow})] \quad (5)$$

where r_o is the intrinsic fluorescence anisotropy, φ_{fast} and φ_{slow} are the fast and slow rotational correlation times; and β_{fast} and β_{slow} are the amplitudes associated with fast and slow rotational time.

The global (slow) rotational correlation time (φ_{slow}) is related to viscosity (η) and molecular volume (V) by the Stokes-Einstein relationship as follow:

$$\varphi_{slow} = \eta V/kT \quad (6)$$

$$V=4/3\pi R_h^3 \quad (7)$$

where R_h is the hydrodynamic radius of the molecule.

Chapter 2

Results and Conclusion

1. Results

1.2 Membrane-induced α– helical structure conformation of α–synuclein:

To monitor the conformational transition from an intrinsically disordered state to a highly helical state upon binding to negatively charged lipids, CD spectroscopic studies were employed. This was already carried out by Neha Jain and Karishma Bhasne.[29, 30] CD was employed to assess the effects of the Cys mutations and to characterize membrane-induced secondary structural changes for all the mutants. In pH 7.4 buffer solution (10mM HEPES, 50mM NaCl), CD spectra for wild-type and Cys mutant shows a minimum near 200 nm that is consistent with random coil configurations (Figure 2(a)). The CD spectra for the Cys mutant were not showing any secondary structural changes compare to wild-type protein, indicating that this change in amino acid does not change the native structure of protein.

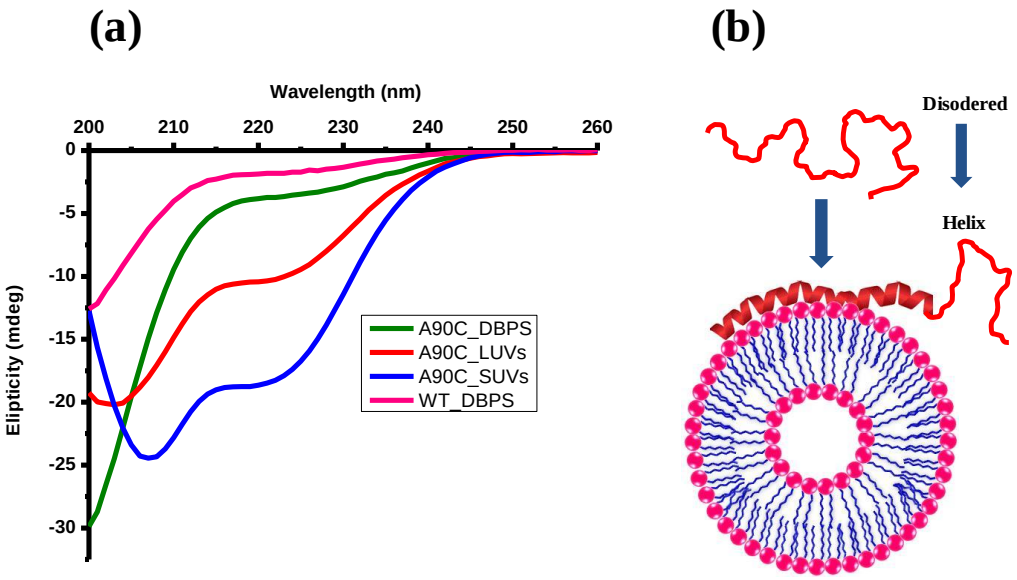

Figure 2.CD spectra of α-synuclein.(a) CD spectra of wild-type (WT) (Pink) and cysteine mutant (Green) and Change in secondary structure from random coil (Green: Native) to α–helix with SUVs(Blue) and LUVs(Red) (b) Schematic diagram to show the transition from disorder form to helical state.

In the presence of anionic phospholipids vesicles (SUVs & LUVs derived from POPG), we observed a transition from random coil state to a highly helical state upon binding with membrane (Figure 2(b)). We have chosen POPG SUVs & LUVs, since it is known to bind to α-synuclein with higher affinity.[29,30] This preliminary result prompted us to understand this conformational change in residue specific manner.

1.2 Site-specific conformational dynamics of membrane-bound α-synuclein:

Site-specific interactions of alpha-synuclein with phospholipid vesicles were monitored by measuring steady-state and time-resolved AEDANS fluorescence anisotropy. The above results from CD and previous time-resolved Trp fluorescence studies from the lab done by Neha Jain lead us to perform further experiments to understand the residue specific conformational dynamics of α-synuclein in membrane-bound state using long lifetime fluorescence probe IAEDANS.

Non-occurrence of cysteine in α-synuclein gives us an advantage. So, 6 single-Cys mutants were generated over the polypeptide chain length (done by Karshima). For incorporating Cys, the residue positions at 9, 18, 56, 78, 90 and 140 were chosen based on earlier reports.[32] Steady-state anisotropy was low (0.017 ± 0.001) for the Cys variants in the free form, which indicates the protein, exist in the disordered form. In the presence of lipids anisotropy was high (0.06 ± 0.01), which indicates that protein is bound to membrane.

We have carried out picoseconds time-resolved fluorescence anisotropy measurements that provide insights into the local and global rotational dynamics of proteins.[33, 34] Global dynamics is represented by a slow rotational correlation time (φ_{slow}) that is generally related to the size (hydrodynamic radius) of the protein (Eq. 5-7). In the unbound form, α-synuclein showed two rotational correlation times. The fast rotational correlation time, φ_1 is 0.34 ns for A90C (Table 1) which represents the local dynamics of AEDANS, whereas φ_2 is 2.2 ns, indicates the segmental mobility which does not depend on the protein size, since the segmental conformation fluctuations depolarize the fluorescence much more rapidly than the global tumbling of the polypeptide chain. For the membrane bound variant of the protein, three rotational correlation times were observed. Rotational correlation time φ_2 and φ_3 indicates the timescale as was observed in the free form. The slowest rotational correlation time, φ_1 is 64 ns, indicates the translational and diffusion

motion of protein over the membrane. This result indicates that α-synuclein is tightly bound to the membrane near 90th amino acid. However, experiments need to be repeated to get the correct rotational correlation time as chi-Square values are high. The anisotropy decay for A90C in the membrane-bound form did not completely depolarize in the timescale of AEDANS fluorescence. The tumbling of SUV-protein complex is much slower (on the μs timescale, estimated from the size of SUVs). Whereas, in the unbound form, anisotropy of AEDANS decayed to zero indicating fast tumbling of protein (Figure 3).

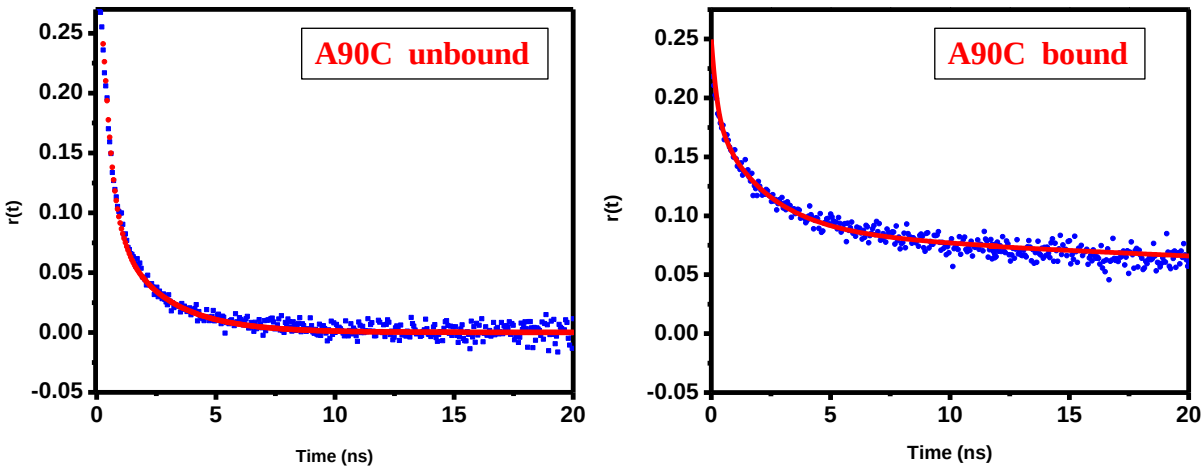

Figure 3.Picosecond time-resolved fluorescence anisotropy decays r (t) in the absence and presence of POPG SUVs of A90C. The solid line (Red) is the bi-exponential fit and tri-exponential fit respectively.

Table 1.IAEDANS fluorescence lifetime and rotational correlation time for single-Cys mutant of α-synuclein (A90C) in the absence and presence of lipid membranes.

Cys Variants (Condition)	Fluorescence Lifetime in ns (amplitude)		Mean Lifetime in (ns)	Chi square value	Rotational Correlation time in ns (amplitude)			Initial anisotropy	Steady state anisotropy	Chi square value
	τ_1 (α_1)	τ_2 (α_2)	τ_{av}	χ^2	φ_1 (β_1)	φ_2 (β_2)	φ_3 (β_3)	r_0	r_{ss}	χ^2
A90C (without SUVs)	**10.13 (0.28)**	**13.64 (0.72)**	**12.64**	**1.08**	**2.2 (0.31)**	**0.34 (0.69)**	**---**	**0.3**	**0.02**	**1.92**
A90C with SUV's	**10.13 (0.28)**	**13.64 (0.72)**	**12.64**	**1.08**	**64.1 (0.3)**	**2.25 (0.3)**	**0.23 (0.4)**	**0.36**	**0.091**	**2.3**

1.3 GdmCl-induced unfolding of Human β_2-microglobulin:

Tryptophan (Trp) fluorescence is highly sensitive to its local environment, and thus often used as a reporter group for protein conformational changes. As surrounding polarity increases, the emission maximum shifts to the lower energy side because of the lowering of the energy level of the fluorescence emission state by stronger dipole–dipole interactions.[33] This fluorescence property is a useful indicator for the conformational change in proteins. β_2m contains two Trp residues at position 60 (exposed) and 95 (partially buried) (Figure 4(b)).[26] We first investigate the changes in intrinsic Trp fluorescence of β_2m from the native to a GdmCl-induced unfolded state. Under the native condition, Trp (W60) fluorescence showed a peak at 350 nm that corresponds to fully exposed tryptophan. As the GdmCl concentration increases to 1 M, there was a decrease in Trp fluorescence intensity, whereas, no shift in the λ_{max} was observed (Figure 4(c)). Further increase in GdmCl concentration till 2 M showed a blue shift in emission maximum which suggest that there are structural changes in β_2m at 60^{th} position while unfolding. This region is getting buried in unfolding process and again getting exposed to aqueous environment at higher concentrations of GdmCl. The fluorescence intensity was reduced further and the maximum wavelength shifted to 345 nm.

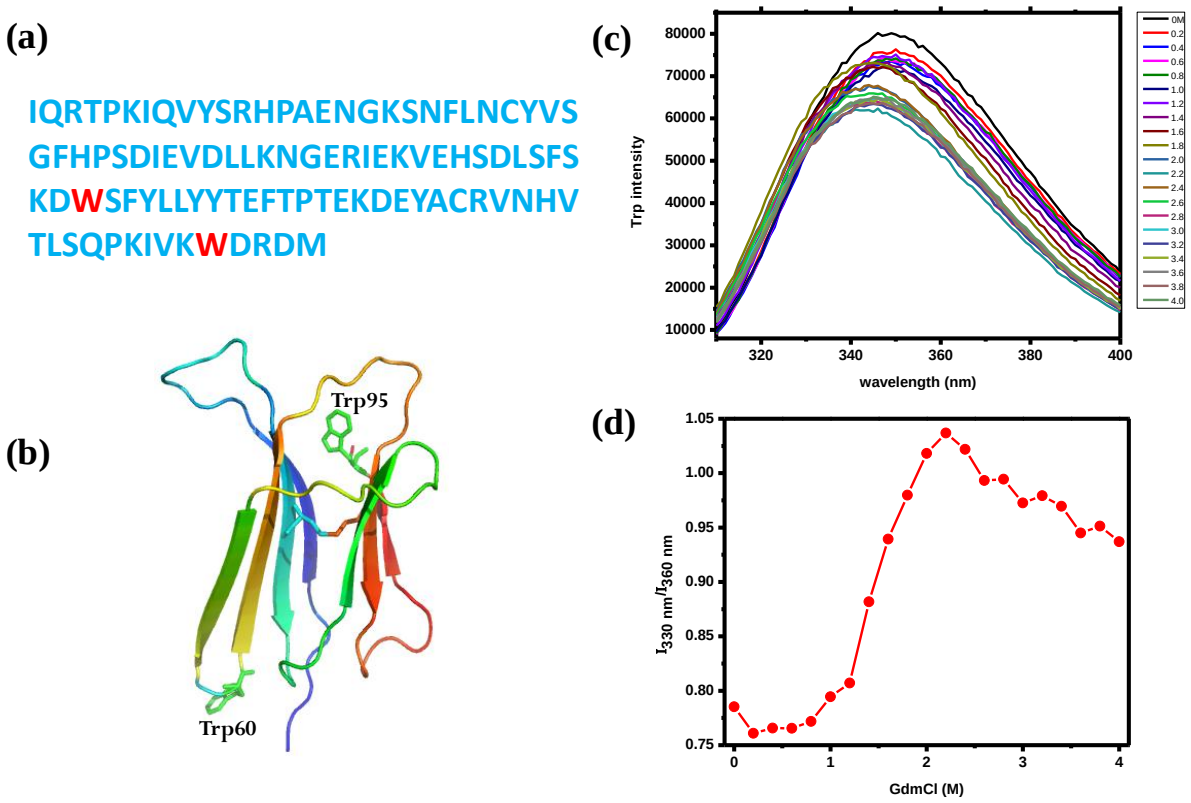

Figure 4.(a) Sequence of β_2m (b) Ribbon diagram of β_2m generating using PyMol (Delano Scientific LLC, CA) from the Protein Data Bank (PDB ID: 1LDS). (c) Fluorescence emission spectra of β_2m (W60) as function of increasing concentration of GdmCl (d) intensity ratios at 330nm and 360nm of β_2m (W60) as a function of increasing concentration of GdmCl.

1.4 Thionitrobenzoate (TNB) quenches the fluorescence of tryptophan in a distance-dependent manner upon unfolding:

The absorbance spectrum of thionitrobenzoate (TNB) overlaps with the emission spectrum of tryptophan (Trp) (forming a FRET pair), and this has been used to measure distance changes during conformational transitions of proteins.[35] β_2m has two tryptophan residues located in two different positions- 60th & 95th. In this study, Trp residue at 60th position (W60) served as the fluorescence donor (D) and a TNB adduct attached via a thiol of an engineered single cysteine variant at N-terminal (W60 NTC-TNB) served as the FRET acceptor (A) (Figure 5 (a)). The fluorescence of Trp is quenched dramatically in the native state of W60 NTC-TNB and is also quenched to a lesser extent in the corresponding unfolded state (Figure 5 (b) & (c)) The observation that the extent of quenching of Trp fluorescence depends on the position of TNB in the protein, indicates that the quenching is distance-dependent.

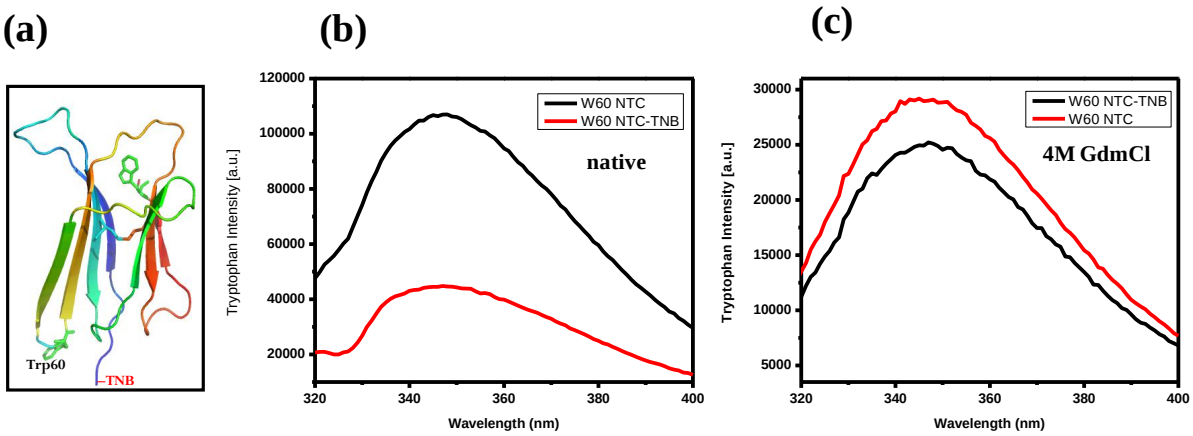

Figure 5.(a) Structure of β_2m the location of W60 and cysteine shown along with the TNB. The sole thiol moiety was labelled with TNB that quenches the fluorescence of Trp in a distance-dependent manner. The TNB-labelled protein named as W60 NTC-TNB. Structure was drawn from PDB file by using the program PyMOL. (b) Fluorescence emission spectra of unlabelled and TNB-labelled protein in native condition. (c) Fluorescence emission spectra of unlabelled and TNB-labelled protein in 4 M GdmCl.

To monitor the GdmCl-induced unfolding using FRET, the changes in the fluorescence of the donor alone (F_D) and donor- acceptor (F_{DA}) proteins were compared (Eq. 2). Figure 6 show the equilibrium unfolding transitions of the unlabeled and TNB-labelled proteins as monitored by change in FRET efficiencies. The fluorescence intensity increases during the unfolding reaction because the D-A distance is expected to increase as the protein unfolds. Hence, FRET efficiencies decreases with an increase in GdmCl concentration and showed mid-point of transition C_m 1.19 M (Figure 6). Previous studies from the lab (done by Dominic Narang), we observed the C_m 1.9 from far-UV CD data and fluorescence emission of β_2m W95. These differences in the C_m values and steady-state fluorescence studies consistently demonstrate the possibility of an intermediate whose conformation is different from the native and unfolded state during the unfolding process.

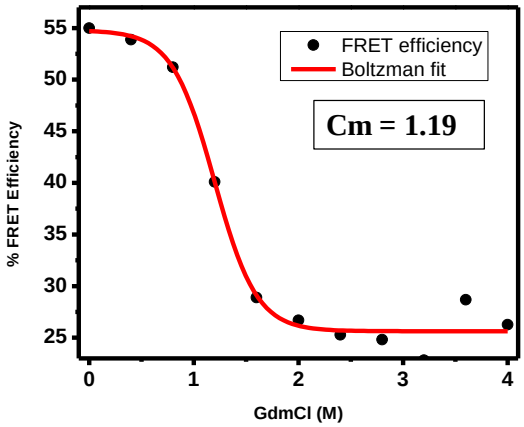

Figure 6.FRET efficiency is plotted against GdmCl concentration. The date for unlabelled and TNB-labelled protein is collected at the emission wavelength of 350 nm after excitation at 295 nm. The red continues line through the data represent the Boltzman fits to a two-sate native ↔ unfolded model.

2.2 Conclusion and Future outlooks:

This work presents structural and dynamical insights into the two amyloidogenic proteins. α-synuclein adopts a α-helical structure when it binds to lipid membranes. However the high resolution structural and dynamical insight of the membrane bound structure of α-synuclein was not fully understood. Our result showed the different degrees of conformational organization of α-synuclein upon biding to membranes. Further region-specific binding and folding studies of α-synuclein to the membrane surface can elucidate the conformational dynamics of α-synuclein. Additionally, this works also highlights the importance of studying a well-characterized model amyloid-forming protein using fluorescence methods to discern the conformational changes during GdmCl-induced unfolding. A variety of different fluorescence techniques have contributed to these findings, demonstrating the importance of fluorescent methods in the study of amyloidogenesis and membrane interactions. We believe that a combination of fluorescence techniques will be extremely useful to delineate the structural and dynamical signatures of amyloidogenic proteins.

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